The Metabolic Response of Cultured Tomato Cells to Low Oxygen Stress

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ABSTRACT

The storage of fruits and vegetables under controlled atmosphere can induce low oxygen stress which can lead to postharvest losses through the induction of disorders like core breakdown and browning. To gain better understanding of the metabolic response of plant organs to low oxygen, cultured tomato cells (*Lycopersicum esculentum*) were used as a model system to study the metabolic stress response to low oxygen (0 and 1 kPa O_2). By adding ^{13}C labelled glucose, changes in the levels of polar metabolites as well as their ^{13}C label accumulation were quantified. Low oxygen stress altered the metabolite profile of tomato cells with the accumulation of the intermediates of glycolysis in addition to increases in lactate and sugar alcohols. ^{13}C label data showed reduced label accumulation in almost all metabolites except lactate and some sugar alcohols. The results showed that low oxygen stress in tomato cell culture activated fermentative metabolism and sugar alcohol synthesis while inhibiting the activity of the TCA cycle and the biosynthesis of metabolites whose precursors are derived from the central metabolism including fluxes to most organic acids, amino acids and sugars.

Keywords: *Lycopersicum esculentum*, ^{13}C label, low O_2 stress, metabolome, cell culture

INTRODUCTION

Upon harvest, some fruits and vegetables are kept under controlled atmosphere (CA) conditions to prolong their storage life while preserving their quality attributes. For instance, in the USA, mature green tomatoes are picked and kept in an environment of 3-5 % oxygen (O_2) and/or 0-3 % carbon dioxide (CO_2) (Kader 2002), as well as temperatures above 10 °C to delay ripening and avoid chilling injury (Kader 2002). Storage life, under CA, is extended by limiting the metabolic activity of the crops through reducing respiration rate which is mostly achieved by the use of low temperatures (Kader and Ben-Yehoshua 2000; Kader 2002). However under the low O_2 conditions used in CA storage, metabolism can switch from respiration to fermentation (which enables the continuation of glycolysis and substrate-level phosphorylation to produce energy in the form of ATP to ensure survival) which can lead to the production of off-flavors leading to loss of quality. As a result there is the need to understand the adaptive mechanisms fruits and vegetables undergo in order to develop better CA conditions to avoid the induction of fermentative metabolism. Also the dependence of man on plant based foods means that the strategies involved in the response of plants to other biotic and abiotic stressors needs to be well understood so that crops which have longer storage lives under CA can be produced (Hertog et al. 2011; Rios-Estepa and Lange 2007; Sweetlove et al. 2003).

The response to stress by plants involves making alterations to their gene expression levels as well as their proteomic and metabolic composition. The accumulation of some amino acids like proline and alanine (van Dongen et al. 2003; van Dongen et al. 2009; Drew 1997; Geigenberger et al. 2000; Reggiani et al. 1993; Thomson and Greenway 1991) along with decreases in protein synthesis (Bailey-Serres and Freeling 1990; Drew 1997) and the dissociation of polysomes (Bailey-Serres and Freeling 1990) are some of the changes that have been noted in plants under various O_2 stress situations. The activity of some enzymes, especially those involved in anaerobiosis like alcohol dehydrogenase and pyruvate dehydrogenase complex have been observed to increase under low O_2 conditions (Andrews et al. 1994; Sachs et al. 1996). Changes in the levels of metabolites and proteins have been observed in some fruits and vegetables kept under CA. In peach fruit kept in an O_2 free environment, induction of fermentation was accompanied by increase in activities of malic enzyme and pyruvate-phosphate dikinase (Lara et al. 2011). Low O_2 induced browning in pears has been shown to be accompanied by a reduction in the levels of metabolites involved

in radical O_2 scavenging (Franck et al. 2007) and respiratory metabolism (Pedreschi et al. 2009). Proteomic analysis of the brown tissues also revealed decreases in the expression levels of proteins involved in energy and antioxidant metabolism (Pedreschi et al. 2007) with an up-regulation and inhibition of transketolase and polygalacturonase, respectively. This shows that the response of plants to stress is well coordinated among the different organisational levels. Even within a single fruit, uneven gas distribution creates low O_2 conditions towards the centre of the fruit inducing anaerobic metabolism leading to the accumulation of GABA and other indicators of low O_2 stress (Biais et al. 2010).

Metabolomic studies on the effect of low O_2 on plants have been carried out in fruits like apples (Lee et al. 2011), peaches (Lara et al. 2011) and pears (Pedreschi et al. 2009). Besides fruits, studies on metabolic changes resulting from the effects of reducing O_2 levels have also been carried out on other plant organs like seedlings (van Dongen et al. 2009) and tissue disks (Geigenberger et al. 2000). The metabolome studies on fruits and seedlings were carried out without ^{13}C label feeding. The incorporation of ^{13}C label into intact plants, fruits or vegetables and the quantification of their label accumulation is a non-trivial task due to the abundance of storage sugars and polymers and the long times needed for these polymers to turn-over and incorporate quantifiable amounts of label into their metabolite pools (Alonso et al. 2007). However, considering the deleterious effect of low O_2 stress on plants, it is important to combine metabolome analysis with ^{13}C label information to give a better insight to the response of plants to O_2 stress. Our interest goes towards better understanding the metabolic responses of fruits and vegetables when exposed to low O_2 conditions as imposed during CA storage. The changes that occur in plant organs due to low O_2 stress can be studied at different organizational levels. Even though, eventually, we are interested in the metabolic response of whole plant organs (fruits and vegetables) proper interpretation of experimental results at such organization level is often hampered by the large degree of tissue differentiation, the complex sink source relations within plant organ, and the huge biological variation involved. In this study we focused on tomato as this is an important model system in plant physiological and molecular research. To reduce the overall complexity of the experimental system, cultured cells were taken as the model system as they can be easily manipulated to grow on a defined media (Schneegurt and McDaniel 1986). As cell culturing can be largely standardized, biological variation can be minimized. Finally, by working with cell suspension cultures the

door is open for direct manipulations, such as the introduction of ^{13}C labeled substrate (glucose) in the growth media.

The objective of this work is to study the metabolic changes that occur in a tomato cell suspension culture when exposed to low O_2 conditions, similar to the conditions experienced by food crops stored under CA. Data on the metabolome was complemented by ^{13}C labeling incorporation. By combining the changes in the metabolome with ^{13}C label incorporation, it becomes possible to recognize which pathways are being up-or down-regulated, something which is not possible when relying on metabolite analysis alone. By monitoring the fast changes as a function of time, insight is created in the metabolic flexibility of the tomato cells in establishing a 'new' metabolic and isotopic steady-state after the perturbation induced by the low O_2 stress. Eventually, this knowledge will be used to enhance our understanding of what is happening at the intact fruit level.

MATERIAL AND METHODS

Cell suspension culture

Cell suspension culture of tomatoes (*Lycopersicum esculentum* var cerasiforme) was established from friable callus grown in the dark at 25 °C. Calli were induced from the leaves of tomatoes grown in a greenhouse. The growth medium of the callus and cells consisted of Murashige and Skoog basal medium (Sigma, Belgium) containing glucose (166 mM) as the carbon source. The media was supplemented with 1000x Nitsch's vitamin mixture (Duchefa, The Netherlands), 9 µM 2,4-dichlorophenoxyacetic acid (Sigma, Belgium) and 11.4 µM benzylaminopurine (Sigma, Belgium) at a pH of 6.0 (Duran-Vila et al. 1995). For callus growth, 7 g of plant growth agar (Duchefa, The Netherlands) was added to the growth media. Cell suspension cultures were incubated in the dark on a rotary shaker at 100 rpm and sub-cultured every six days with the ratio of medium to cells kept at 3:1. Cell viability was determined periodically using the Evans blue exclusion method (Puschmann and Romani, 1983).

Bioreactor experiments

For low O_2 stress studies, 250 mL cell suspension culture in the log phase of growth (five days after sub-culturing as determined during a preliminary study), were transferred to a

Lambda Minifor bench-top laboratory bioreactor (Lambda Laboratory Instruments, Czech Republic) maintained at a temperature of 25 °C. The concentration of glucose in the medium at the time of transfer was 85 mM. The pH of the medium was kept at 6 throughout the experiment and low O_2 stress was achieved by bubbling gas through the medium at a rate of 10 L/h for the entire duration of the experiment. Four independent replicate experiments per condition were carried out. The cells were allowed to adapt to the prevailing O_2 condition during the first four hours, after which a substrate pulse (10 mL volume) containing ^{13}C label was added to bring the concentration of glucose to 166 mM without significantly changing the ratio of medium to cells (3:1). The concentration of ^{13}C label was adjusted to ensure that 50 % of the total glucose in the medium was U-^{13}C glucose (uniformly labelled ^{13}C glucose). Cell samples were taken every hour for the next eight hours and a final sample taken 24 hours from the start of the experiment. Sampling was carried out by withdrawing 15 mL of medium containing cells from the bioreactor. The samples were washed with glucose free medium, snap frozen in liquid nitrogen and stored at -80 °C. The samples were lyophilized (Duratop and Duradry, FTS Systems Inc., Stone Ridge, NY, USA) for 24 h and dried for further 24 h in an oven at 50 °C. The experimental conditions tested were 0, 1 and 21 kPa O_2 and these have been selected based on specific measurements as will be detailed below.

Analysis of primary metabolite in cultured tomato cells

Samples of lyophilized cells (10 mg) were extracted at 70 °C for 15 min with 700 µL of methanol containing 45 µL of 291 ng/µL internal standard (phenyl-β-D-glucopyranoside in methanol). An equal volume of water was added and vortexed rigorously after which 325 µL chloroform was added to separate the non-polar fraction. The mixture was centrifuged for 5 min at 14,000 rpm and 500 µL of the polar fraction was dried under a stream of nitrogen. The dried residue was oximated by adding 40 µL of 20 mg/mL methoxyamine hydrochloride in pyridine and incubated at 37 °C for 90 min. Derivatisation was carried out at 37 °C for 30 min after adding 60 µL N-methyl-N-(trimethylsilyl)trifluoroacetamide (Oms-Oliu et al. 2011, Roessner et al. 2000).

Metabolite separation was carried out on a GC-MS system which consisted of a 7890A GC (Agilent Technologies, Palo Alto, CA, USA) and 5975C VLMSD MS with triple-Axis detector (Agilent Technologies, Palo Alto, CA, USA). The analysis was performed on a 30 m HP-5MS column with 0.25 mm internal diameter and 0.25 µm film thickness (Agilent

Technologies, Palo Alto, CA, USA) with a split ratio of 10:1. The sample inlet and interface temperatures were set at 230 °C and 250 °C, respectively, while the ion source was adjusted to 230 °C. The oven temperature program consisted of 1 min heating at 50 °C followed by a ramp of 10 °C/min to a final temperature of 310 °C with further holding of 13 min. The system was then temperature equilibrated for 5 min at 70 °C before injection of the next sample. Helium was used as the gas carrier at a constant flow rate of 1 mL/min and the GC-MS program was retention time locked to tetracosane (Agilent Technologies Inc., Wilmington, USA).

Metabolite quantification, ^{13}C label determination and statistical analysis

All GC-MS chromatograms and mass spectra were evaluated and deconvoluted using the Chemstation (Agilent Technologies Inc., Wilmington, USA) and AMDIS (automated mass spectral deconvolution and identification system) software. Metabolites were identified by comparing their retention times and mass spectra with an in-house library and confirmed with the Agilent Fiehn GC-MS Metabolomics RTL library (Agilent Technologies Inc., Wilmington, USA). The abundance of each metabolite was normalized by the sample weight as well as the peak area of the internal standard. Where available, the ^{13}C label accumulated by the metabolite was calculated from the molecular ion fragment and corrected for the presence of naturally occurring isotopes (Wahl et al. 2004).

Univariate statistical analysis was performed to identify the difference between the levels of the metabolites under the three O_2 levels at the different time points using Origin 8 (v 8.0725, OriginLab Corporation, Northampton, USA). The difference among means was identified using the t-test with a significance level of $p = 0.05$. Multivariate statistical analysis was performed in The Unscrambler (v.10.2, CAMO A/S, Trondheim, Norway) using partial least square discriminate analysis (PLS-DA) methods (Norden et al. 2005, Pedreschi et al. 2007) to identify which metabolites change in response to O_2 stress. In PLS-DA, the different O_2 growth conditions were considered categorical Y-variables with the metabolite abundance as X-variables. The data was mean centered and the variables given equal variance.

Determination of O_2 uptake rate of tomato cells

The O_2 uptake rate of the cells was determined using an in-house built biological oxygen monitor (BOM) working on the principle of Clark's O_2 electrode (Lammertyn et al. 2001).

The electrode positioned at the bottom of the BOM (5 mL working volume), consisted of a central platinum disc, where reduction of O_2 occurred, and a reference electrode. Potassium chloride solution (3 M) was used to achieve conduction between the two electrodes. The current through the electrode when applying a charge of 1.6 V was proportional to the O_2 partial pressure in the test system. The current was converted and measured using a data logger (Agilent Technologies, Belgium). Three independent replicate measurements were performed using 4 mL of cell suspension culture from the log phase of growth. Corrections were carried out for the effect of temperature, pressure and salt concentration on the solubility of O_2 in a liquid medium.

RESULTS

Defining experimental conditions

The O_2 levels used in this experiment were chosen so as to limit respiration in the cells. The Michaelis-Menten constant (K_M) of respiration was used as a guide to select the appropriate O_2 levels. The K_M of respiration was determined by measuring the depletion of O_2 within a BOM and calculating the O_2 uptake rate using equation 1.

$$\frac{d[O_2]}{dt} = V_{max} \frac{n \times [O_2]}{K_M + [O_2]} \quad (1)$$

where V_{max} [µg/(L x s x 10^6 cells)] is the maximum O_2 uptake rate and n (10^6 cells) is the number of cells placed in the BOM. Using three independent O_2 depletion experiments, the rate of consumption of O_2 by the cells was estimated along with the K_M of respiration (Fig. 1). A K_M of 0.64 µM corresponding to an O_2 concentration of 0.045 kPa and a V_{max} of 1.805 µg/(L x s x10^6 cells) was obtained. Based on the K_M of respiration, three O_2 levels were chosen for this research: an O_2 concentration below the K_M (0 kPa), an O_2 concentration slightly above the K_M (1 kPa) and 21 kPa O_2 which served as the control.

Early changes to low O_2 stress

The metabolome of cultured tomato cells was affected by the induction of O_2 stress. Using GC-MS analysis, 63 metabolites belonging to the polar metabolite fraction were tentatively identified and quantified in cultured tomato cells through methanolic extraction (Appendix S1). Accumulation of ^{13}C label could be calculated for 47 of these metabolites (Appendix S2).

Different concentrations of dissolved O_2 in the growth medium of cultured tomato cells clearly lead to different levels for most primary metabolites (Fig. 2 and Appendix S1). Sharp increases (more than two fold increase compared to the control) were observed in the levels of fructose-6-phosphate, glucose-6-phosphate, lactate and pyruvate with seemingly steep reduction (about 50 %) in maltose, L-dehydroascorbate and tyrosine within the first hour of cells incubated at 1 and 0 kPa O_2 levels (Fig. 2). Increases in glucose-6-phosphate and fructose-6-phosphate also occurred in cells incubated under control conditions, yet these were significantly less than those observed at the low O_2 conditions. Mannitol and sorbitol also increased significantly within the first hour under both low O_2 stress conditions (Fig. 2). With the exception of erythritol and glycerol-3-phosphate (Appendix S1) which did not change significantly within the first hour under low O_2 stress, almost all the other metabolites decreased. From the second to the fourth hour, all the metabolites continued either their increasing or decreasing trend under low O_2 stress, with the exception of 3-phosphoglycerate and gluconate that started to increase after the third and second hour, respectively.

Effect of adding ^{13}C glucose

The addition of a small amount of media saturated with ^{13}C glucose (at 4 h) changed the levels of metabolites in both the control and the low O_2 stressed cells (Fig. 2 and Appendix S1). In the control cells, the levels of most metabolites started to increase and, after 12 hours of incubation, reached levels higher than the starting point (Appendix S1). However, the levels of β-alanine, benzoate, L-dehydroascorbate and homoserine decreased within one hour after the addition of ^{13}C-labeled glucose (so at 5 h). Some metabolites (benzoate, chlorogenate, erythritol, GABA, galactose, gluconate, glycerol, glycerol-3-phosphate, isoleucine, lactate, maltitol, maltose, mannitol, proline, quinate, ribose, sorbitol, stearate and urea) were less abundant after 12 h of incubation compared to 0 h. In the low O_2 stressed cells, the response to the extra glucose included a reduction in the rate of decline of those metabolites which were already decreasing within the first four hours of O_2 stress. Small increases in the levels of some metabolites (alanine, cysteine, galactose, glutamate, glutamine, isoleucine, leucine, phenylalanine, succinate, stearate, trehalose and urea) were observed between 5 h and 6 h, even though these metabolites started to decrease thereafter (Appendix S1). The levels of metabolites like norleucine, and mannose seem not to be affected by the induction of low O_2 stress nor by the addition of ^{13}C glucose.

Fast ^{13}C label incorporation

^{13}C label was incorporated into the different metabolites after the addition of ^{13}C glucose at 4 h (Fig. 3 and Appendix S2). In the control cells, ^{13}C label could be detected in all metabolites one hour after the addition. For the metabolites involved in glycolysis, a fast build-up of ^{13}C label was observed, reaching steady state labeling levels after a few hours. ^{13}C label accumulation by other metabolites occurred more slowly often not reaching steady state levels within the timeframe of the experiment. The rate of ^{13}C label accumulation into the different metabolites was dependent on the metabolic proximity to the supplied substrate and the need for the cell to synthesize or utilize the metabolites involved. For instance, glucose-6-phosphate and 3-phosphoglycerate (Fig. 3) were labeled close to the maximum level of 50 % within three hours after the label addition while isoleucine apparently even had not reached its maximum labeling eight hours after the addition. Low O_2 stress affected the accumulation of ^{13}C label by the cells. Compared to the control, the accumulation of ^{13}C label in most metabolites was low under both low O_2 stress conditions. Metabolites which, compared to the control, accumulated more or about the same ^{13}C label under both low O_2 stress conditions included glycerate, glycerol, glycerol-3-phosphate, lactate, mannitol, ribose and sorbitol (Fig. 3).

Comparing the two low O_2 stress conditions, the metabolites which were more abundant under 0 kPa O_2 compared to 1 kPa O_2 in more than seven sampling time points before and after the addition U-^{13}C glucose included 3-phosphoglycerate, fructose-6-phosphate, glucose-6-phosphate, glycerol, glycerol-3-phosphate, lactate, maltitol, pyruvate and ribose (Fig. 2). Similarly, 2-oxoglutarate, citrate, chlorogenate, isoleucine, leucine, malonate, succinate, serine and shikimate were more abundant in 1 kPa than 0 kPa O_2 in more than seven sampling time points (Appendix S1). No metabolite accumulated significantly higher ^{13}C label under 0 kPa O_2 compared to 1 kPa O_2 in more than seven time points (Appendix S2). Only GABA, glutamate, fructose-6-phosphate and glucose-6-phosphate accumulated more ^{13}C label under 1 kPa O_2 compared to 0 kPa in more than seven sampling time points (Appendix S2).

Changes after 24 hours of O_2 stress

The metabolites continued to change when incubated for a further 12 hours under low O_2 stress (Fig. 2 and Appendix S1). With the exception of the intermediates of glycolysis, glycerol-3-phosphate, lactate, maltitol, mannitol, sorbitol and ribose which increased and

reached peak levels within the first twelve hours (Fig. 2), all other metabolites continued to decline under O_2 stress. With respect to ^{13}C label accumulation, cells exposed to low O_2 stress continued to increase their ^{13}C label content, albeit slowly except for sucrose, lactate and urea (Fig. 3 and Appendix S2). Under 21 kPa O_2, no further increase in ^{13}C label was observed in the metabolites involved in the central metabolism (3-phosphoglycerate, citrate, fructose-6-phosphate, fumarate, glucose-6-phosphate, malate and succinate), indicating that isotopic steady-state had been reached within 24 hours of incubation. The ongoing accumulation of ^{13}C label by cells exposed to the two low O_2 stress conditions shows that isotopic steady-state was not reached within the experimental duration. A metabolic network map showing the effects of low O_2 stress on the central metabolism and associated pathways in cultured tomato cells after 24 hours of incubation can be seen in Fig. 4.

Global metabolomic changes in tomato cells due to O_2 stress

The relationship between metabolite abundance, O_2 level and incubation time was evaluated to identify those metabolites that play a key role in the low O_2 stress response using PLS-DA. A plot of metabolite and treatment correlation loadings superimposed on the sample scores can be found in Fig. 5. PLS-DA aims to reduce the multivariate metabolite space into a low dimensional space of latent variables (LV; the axes in Fig. 5) while regressing these on the treatment variables (O_2 level and time) thus facilitating interpretation of the data. The positions of the individual samples in this new space are represented by the colored symbols. Their position can be interpreted both in relation to the position of the metabolites (the open symbols) and in relation to the direction of time and the three O_2 levels applied (represented by the four arrows). Fig. 5A is a plot of LV1 vs. LV2 while Fig. 5B is a plot of LV1 vs. LV3 for the same PLS-DA analysis. These first three LVs cover about 82 % of the observed variation in metabolite levels with the regression explaining 81 % of the variation in the treatment variables. Fig. 5 therefore provides a good overview of the changes occurring in the 63 dimensional metabolite space. Metabolites oriented in Fig. 5 in the same direction are positively correlated and show similar response in time to a given O_2 treatment or incubation time, while those in opposite directions are defined as negatively correlated showing opposite response to a given O_2 treatment or incubation time (in terms of an increase or decrease). PLS-DA revealed a divergence of tomato cell metabolome with increasing incubation time in response to O_2 stress. Separation between the control (21 kPa O_2) and the two low O_2 conditions could be achieved using the first two latent variables (Fig. 5A). Together these two

latent variables accounted for 78 and 47 % of the explained X and Y-variance, respectively. A third latent variable (Fig. 5B) was needed to achieve a clear separation between the two low O_2 conditions (1 and 0 kPa). Using the first three latent variables the difference between the control and the two low O_2 conditions could be explained with a total X and Y-variance of 82 and 81 %, respectively.

The arrows in Fig. 5 show how the experimental conditions (O_2 level and incubation time) correlate with the metabolites in the new parameter space. Metabolites which are found around a given O_2 level indicate a close association with that O_2 condition. The metabolites which were closely associated with the low O_2 conditions included 3-phosphoglycerate, fructose-6-phosphate, glucose-6-phosphate, glycerol, glycerol-3-phosphate, lactate, mannitol, maltitol, pyruvate, ribose and sorbitol (Fig. 5A). All these metabolites increased with the induction of O_2 stress. With respect to time (Fig. 5A) metabolites above the horizontal axis increased with incubation time while those below decreased with incubation. For example, 3-phosphoglycerate and methionine, associated with the low O_2 levels and the control respectively, increased with incubation time while benzoate and gluconate which are located opposite the time arrow but associated with the control and the low O_2 levels respectively, decreased with incubation time. In Fig. 5B, the direction of the time arrow shows that the changing levels of the metabolites under the different O_2 levels is largely independent of time and mainly a direct result of the O_2 level applied.

The variable importance in projection scores (VIPs) were calculated and used to assess the contribution of each metabolite in explaining the effects of incubation time and the different O_2 levels and thus being responsible for the observed separation in Fig. 5. Metabolites with VIP close to or greater than one are considered important in given model while those with significantly less than one are less important and can be excluded from the model.

Table 1 shows a list of the ten highest VIP for each O_2 treatment and incubation time. The VIP scores showed that benzoate, citramalate, fructose, glycerol-3-phosphate, gluconate, homoserine, leucine, norleucine pyroglutamate, sorbitol, tyrosine and valine were the major metabolites which explained the difference between the control and the low O_2 levels. These metabolites, with the exception of glycerol-3-phosphate, were present in higher amounts in the control compared to the low O_2 incubated cells. Gluconate which decreased in abundance under the three O_2 levels had the highest VIP for incubation time.

DISCUSSION

The application of metabolomics to understand the response of plant to low O_2 and other abiotic perturbations has gained tremendous attention in the last decade (Shulaev et al. 2008, Obata and Fernie 2012). By combining metabolome measurements with ^{13}C labeling data, metabolic responses can be interpreted in terms of changed fluxes through the involved pathways. In this study, metabolomics and ^{13}C labeling data were combined to investigate the response of tomato cell suspension culture to a sharp decrease in O_2 levels. The experimental conditions were selected to mimic the O_2 levels experienced by fruits and vegetables transferred to CA storage.

Using cell suspension cultures results in highly repeatable results.

In this study cultured tomato cells were taken as the model system thus to exclude diffusion barriers, as would be encountered in intact tissue, and to enable the direct manipulation of substrate levels (both ^{13}C glucose and O_2). By taking away these diffusion barriers, any variation related to the typical fruit-to-fruit variation in tissue porosity has been cancelled out. In addition, biological variation between cell suspension cultures are assumed to be relatively small as the culturing conditions are tightly controlled. This is mirrored by the small error bars in Fig. 2 and 3, representing the variation based on 4 independent replicate experiments, and also by the tight clustering of the replicate observations in Fig. 5).

The response to low O_2 stress involves changes in the overall metabolism of plant cells

Low O_2 stress was accompanied by an increase in the levels of some metabolites (3-phosphoglycerate, fructose-6-phosphate, glucose-6-phosphate, glycerol, glycerol-3-phosphate, lactate, mannitol, pyruvate, ribose and sorbitol) while other metabolites decreased. The increase in the level of glycerol, glycerol-3-phosphate, lactate and the sugar alcohols (with the exception of mannitol) was accompanied by an increase in ^{13}C label accumulation, which indicates an increased flux due to an increased synthesis. Among the metabolites which decreased under low O_2 stress were some within the general group of sugars (except ribose and the sugar phosphates), amino acids and organic acids (except lactate). In addition to their decreased levels, these metabolites accumulated less ^{13}C label which suggests a general reduction of their synthesis. The up and down regulation of the various metabolite levels, in

366 combination with the differential accumulation of ^{13}C label shows that the response of the
367 plants to low O_2 stress is not limited to a particular pathway but involves changes in the
368 overall metabolism of cells.

369 **Low O_2 stress is accompanied by the accumulation of glycolytic intermediates, activation**
370 **of fermentative metabolism and decrease in TCA cycle activity**

371 Low O_2 stress in cultured tomatoes cells resulted in the accumulation of the intermediates of
372 glycolysis, which shows an inhibition of pathways downstream of glycolysis. The
373 accumulation of glycolytic intermediates with low O_2 stress has also been observed in other
374 plant systems (Galili 2011, Miyashita and Good 2008). The increased levels of glycolytic
375 intermediates under low O_2 stress without a corresponding increase in ^{13}C label (Fig. 4)
376 suggests a reduction of the uptake rate labelled substrate by the cells and an increased release
377 from unlabelled intracellular sources. Suppression of substrate uptake by cells could be a
378 strategy to reduce energy expenditure since substrate uptake is an active transport process
379 which consumes cellular energy. The ability of plants to reduce energy consumption in the
380 form of ATP is a an important requirement to ensure their survival under low O_2 conditions
381 (Drew 1997, Geigenberger et al. 2000, Geigenberger 2003, Gupta et al. 2009). In potato
382 tubers incubated under ^{14}C sucrose, a reduction in substrate uptake was observed within 2 h of
383 incubation when the level of dissolved O_2 was reduced below 12 kPa (Geigenberger et al.
384 2000). In *Arabidopsis* roots grown under O_2 limiting conditions, a reduction in the transcripts
385 encoding for proteins involved in energy consuming processes like transport and biosynthesis
386 was observed (van Dongen et al. 2009).

387 The response of the intermediates of the TCA cycle to low O_2 stress involved a general
388 decrease in levels accompanied by low ^{13}C label accumulation (Appendices S1 and S2),
389 which indicates a reduced activity of the TCA cycle. The decrease in TCA cycle activity
390 under low O_2 stress could be due to a general inhibition of oxidative phosphorylation due to
391 the unavailability of O_2. Taking into account that O_2 serves as an electron acceptor in the
392 oxidation of reduced cofactors (NADH and FADH) produced from the TCA cycle, the
393 absence of O_2 will impede the regeneration of these cofactors eventually leading to an
394 inhibition of the TCA cycle. The increase in gluconate (Appendix S1) during the early stages
395 of low O_2 stress could be due to increases in the activity of the pentose phosphate pathway
396 (PPP). Considering that PPP activity results in the synthesis of reducing equivalents required

to ensure the redox balance of the cell and to produce electrons for the reduction of reactive O_2 species, its activation under low O_2 stress is likely. Increases in the activity of PPP have been noted in cell cultures (Baxter et al. 2007, Ishikawa et al. 2010) and roots (Lehmann et al. 2008) subjected to oxidative stress.

The build-up of lactate (Fig. 2) in the cells due to low O_2 stress indicates a possible shift in energy metabolism from respiration to fermentation. The activation of fermentative metabolism under low O_2 stress is important for producing ATP to ensure the survival of cells due to the inhibition of the TCA cycle. The accumulation of pyruvate (Fig. 2) has been observed when plants switch from respiratory to fermentative metabolism because of the inefficiency of fermentation to utilize the intermediates of glycolysis (Aloni and Rosenshtein 1982, Geigenberger et al. 2000, Geigenberger 2003, Miyashita and Good 2008, Reggiani et al. 1993, van Dongen et al. 2009). The induction of fermentation as a response to O_2 stress is not only restricted to changes in the metabolome, as studies in maize roots have shown increases in the levels and expressions of anaerobic proteins and genes (Sachs et al. 1996). Increase in the expression profile of anaerobic proteins with O_2 stress have also been observed in *Arabidopsis* roots (Klok et al. 2002). Considering that the fermentation of fruits and vegetables can result in the production of off-flavors (Kader and Ben-Yehoshua 2000; Kader 2002), these O_2 levels should be avoided during CA storage.

The adaptive response of plants to low O_2 stress involved reducing the biosynthesis of sugars, amino acids and organic acids

The response to low O_2 stress involved a general reduction in the levels of free amino acids and organic acids (Appendix S1). This decrease could be due to the need to reduce energy consumption by limiting biosynthetic reactions which consumes energy. A general decrease in the levels of some amino acids have also been observed in *Arabidopsis* cells subjected to menadione-induced oxidative stress (Baxter et al. 2007) and in pears (Franck et al. 2005) and apples (Lee et al. 2011) under CA storage. In contrast, the metabolic response of anoxia-intolerant plant tissue like maize roots (Thomson and Greenway 1991, Aloni and Rosenshtein 1982) and anoxia-tolerant species like rice seedlings (Magneschi and Perata 2009) and suspension cultures (Ishikawa et al. 2010) is characterized by increasing levels of some amino acids. The different response of amino acids might suggest a possible role in the adaptive response of plants to low O_2 stress even though this might depend on the tissue under

study and the mode of low O_2 stress induction. Below 1 kPa O_2, glutamate, among the different amino acids, accumulated the highest ^{13}C labeling. This high accumulation of ^{13}C label by glutamate might be related to its role in the synthesis of nucleotides, chlorophylls, polyamines and alkaloids as well as its involvement in the synthesis of other amino acids like glutamine, proline, putrescine and GABA.

Marked reductions were noted in the levels of most sugars with an increase in sugar alcohols under low O_2 stress (Fig. 4). The decrease in levels of sucrose, maltose and trehalose with low O_2 stress could be related to the suppressed substrate uptake requiring alternative sources to generate energy. The only sugar which increased with low O_2 stress, ribose, has also been observed to accumulate in pears (Lee et al. 2011), cell cultures (Baxter et al. 2007) and roots (van Dongen et al. 2009) subjected to different O_2 stress conditions. Even though the precise role of ribose in plants is unknown (besides its role in the synthesis of ADP-ribose for use in the PPP) ribose has been shown to enhance the regeneration of ATP in mammalians cells under anoxia (St. Cyr et al. 1989). It is possible that ribose might play a similar role in plant cells subjected to low O_2 stress. The increase in the levels of sugar alcohols was accompanied by an increase in ^{13}C label (Fig. 4), suggesting an increased synthesis. Sugar alcohol accumulation has been observed as one of the by-products of the low O_2 stress response of plants (Baxter et al. 2007, Ishikawa et al. 2010, Lee et al. 2011, Pedreschi et al. 2009, van Dongen et al. 2009). Sugar alcohol accumulation has also been reported in plant subjected to other abiotic stress conditions (Moing 2000). Sugar alcohol accumulation in response to low O_2 stress might be related to their role in complementing carbon storage reserves during sugar deprivation (Moing 2000).

Organic acids have been noted to play crucial roles in the adaptive response of plants to low O_2 stress. In potato tissues, stepwise decrease in the concentration of O_2 caused changes in the levels of most organic acids (Geigenberger et al. 2000), while the response of culture cells to oxidative stress involved decreases in the levels of some organic acids (Baxter et al. 2007, Ishikawa et al. 2010). In our study, low O_2 stress was characterised by a general decrease in the levels of most organic and fatty acids (Appendix S1). The observed decrease in glycerate levels even though it accumulated about the same amount of ^{13}C label compared to control (Appendix S2) indicates a rather constant synthesis but an increased breakdown. The general reduction in amino and organic acids appears to be in agreement with the survival strategy employed by plants under low O_2 stress which involves reducing anabolic reactions along

with the nitrogen metabolism to make more substrate available for glycolysis (Drew 1997, Galili 2011, Geigenberger 2003, Li et al. 2012). In the case of ripening fruits such low O_2 stress might potentially affect anabolic metabolism, resulting in a reduced availability of sugars, organic acids and amino acids needed to synthesize aroma volatiles (Perez and Sanz 2008; Schwab et al. 1997) eventually reducing the commercial value of fruits.

The levels of other nitrogen containing metabolites were reduced under low O_2 stress

β-alanine, GABA and putrescine all decreased and accumulated less ^{13}C label (Fig. 4) showing a decrease in their synthesis in response to O_2 stress. The role of putrescine in the biosynthesis of polyamines is well documented (Pedreschi et al. 2007, Van de Poel et al. 2012, Ye et al. 1997), showing that a decrease in putrescine synthesis leads to a reduction in the biosynthesis of polyamines. In *Arabidopsis* cells under oxidative stress, decreases in the levels of transcripts related to putrescine were observed along with decreases in concentration (Baxter et al. 2007). In fruits (Pedreschi et al. 2009) and other plant parts (Ye et al. 1997), putrescine has been revealed to increase due to O_2 stress. The accumulation of putrescine in this species under low O_2 stress might help in the maintenance of ionic balance to counter the increase in the levels of some organic acids like GABA (Reggiani et al. 1993). The role of β-alanine, a polyamine product, implicated in the adaptive response of plants to various stressors, including low O_2 stress response is unclear. In Empire apples, low O_2 stress was accompanied by an increase in β-alanine (Lee et al. 2011) even though β-alanine reduction was observed in *Arabidopsis* cells (Baxter et al. 2007).

The response of GABA to low O_2 stress was opposite to that observed in some studies on fruits where GABA has been shown to accumulate (Franck et al. 2005; Lee et al. 2011; Pedreschi et al. 2009). In *Arabidopsis* cell suspension cultures grown under normal and elevated O_2 conditions, the levels of GABA were found to be higher under the elevated O_2 condition (Williams et al. 2008). In *Arabidopsis* cell cultures subjected to menadione induced oxidative stress, there was no significant change in the levels of GABA (Baxter et al. 2007) while a similar treatment on *Arabidopsis* roots resulted in a marked reduction in the levels of GABA (Lehman et al. 2009). Considering that the activation of GABA synthesis have been observed to occur under reduced cytoplasmic pH (Carroll et al 1994), the production of GABA could be linked to the synthesis and/or degradation of other organic acids in cells, a situation which could be both tissue and treatment specific.

CONCLUSIONS

The use of tomato cell suspension cultures proved to be an efficient tool to study the metabolic effects of low O_2 stress on higher plant cells revealing clear changes in the primary metabolites. By combining metabolome data with ^{13}C label information, clear patterns in the response of the cells to low O_2 stress emerged. Low O_2 stress inhibited the biosynthesis of amino acids, organic acids and other nitrogen containing compounds. Under low O_2 stress, accumulation of the intermediates of glycolysis, some sugar alcohols and lactate was observed. The lower the O_2 level, the higher this accumulation. The accumulation of glycolytic intermediates went hand-in-hand with a decreased ^{13}C labeling, indicating a reduced overall flux through glycolysis and not an up-regulation. The accumulation of lactate under low O_2 stress signaled the induction of fermentative metabolism providing an escape for the accumulating pyruvate levels.

The fact that not every intermediate was labeled up to the same steady state level indicates that the various intermediates also received unlabeled inputs from other pathways. The ^{13}C label accumulated by the different metabolites showed that for the control cells (21 kPa O_2) isotopic steady-state of the central metabolism was reached within 12 h while, under low O_2 stress, this took more than 24 h, mirroring the suppressed metabolic rates at low O_2 levels.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Changes in metabolite levels with time following the introduction of low O_2 stress.

Appendix S2. Accumulation of ^{13}C label by the different metabolites of cultured tomato cells following incubation under low O_2 stress.

518 **REFERENCES**

519 Aloni B., Rosenshtein G. (1982) Effect of flooding on tomato cultivars– the relationship
520 between proline accumulation and other morphological and physiological-
521 changes. *Physiologia Plantarum* **56**: 513–517

522 Alonso A.P., Goffman F.D., Ohlrogge J.B., Shachar-Hill Y. (2007) Carbon conversion
523 efficiency and central metabolic fluxes in developing sunflower (*Helianthus annuus* L.)
524 embryos. *Plant Journal* **52**, 296–308.

525 Andrews D.L., MacAlpine D.M., Johnson J.R., Kelley P.M., Cobb B.G., Drew M.C. (1994)
526 Differential induction of mRNAs for the glycolytic and ethanolic fermentative pathways by
527 hypoxia and anoxia in maize seedlings. *Plant Physiology* **106**, 1575–1582.

528 Bailey-Serres J., Freeling M. (1990) Hypoxic stress-induced changes in ribosomes of maize
529 seedling roots. *Plant Physiology* **94**, 1237–1243.

530 Baxter C.J., Redestig H., Schauer N., Repsilber D., Patil K.R., Nielsen J., Selbig J., Liu J.,
531 Fernie A., Sweetlove L.J. (2007) The metabolic response of heterotrophic *Arabidopsis* cells to
532 oxidative stress. *Plant Physiology* **143**, 312–325.

533 Biais B., Beauvoit B., Allwood J.W., Deborde C., Maucourt M., Goodacre R., Rolin D.,
534 Moing A. (2010) Metabolic acclimation to hypoxia revealed by metabolite gradients in melon
535 fruit. *Journal of Plant Physiol*ogy **167**, 242–245.

536 Carroll A.D., Fox G.G., Laurie S., Phillips R., Ratcliffe R.G., Stewart G.R. (1994)
537 Ammonium assimilation and the role of γ-aminobutyric acid in pH homeostasis in carrot cell
538 suspensions. *Plant Physiology*, **106**, 513–520.

539 van Dongen J.T, Schurr U., Pfister M., Geigenberger P. (2003) Phloem metabolism and
540 function have to cope with low internal oxygen. *Plant Physiology*, **131**, 1529–1543.

541 van Dongen J.T., Frohlich A., Ramirez-Aquilar S.J., Schauer N., Fernie A.R., Erban A.,
542 Kopka J., Clark J., Langer A., Geigenberger P. (2009) Transcript and metabolite profiling of
543 the adaptive response to mild decreases in oxygen concentration in the roots of *Arabidopsis*
544 plants. *Annals of Botany*, **103**, 269–280.

545 Drew M.C. (1997) Oxygen deficiency and root metabolism: injury and acclimation under
546 hypoxia and anoxia. *Annual Review of Plant Physiology and Plant Molecular Biology*, **48**,
547 223–250.

548 Duran-Vila N., Carbonell E.A., Perez Boada S., Semancik J.S. (1995) Growth of healthy and
549 viroid-infected tomato cells in vitro. *Plant Science*, **105**, 111–120.

550 Franck C., Lammertyn J., Nicolai B. (2005) Metabolic profiling using GC-MS to study
551 biochemical changes during long-term storage of pears. Acta Horticulturae (ISHS), **682**,
552 1991–1998.

553 Franck C., Lammertyn J., Tri Q.H., Verboven P., Verlinden B., Nicolaï B.M. (2007)
554 Browning disorders in pear fruit. *Postharvest Biology and Technology*, **43**, 1–13.

555 Galili G. (2011) The aspartate-family pathway of plants: linking production of essential amino
556 acids with energy and stress regulation. *Plant Signaling and Behaviour*, **6**, 192–195.

557 Geigenberger P. (2003) Response of plant metabolism to too little oxygen. *Current Opinion*
558 *in Plant Biology*, **6**, 247–256.

559 Geigenberger P., Fernie A.R., Gibon Y., Christ M., Stitt M. (2000) Metabolic activity
560 decreases as an adaptive response to low internal oxygen in growing potato tubers. *The*
561 *Journal of Biological Chemistry*, **381**, 723–740.

562 Hertog M.L.A.T.M., Rudell D.R., Pedreschi R., Schaffer R.J., Geeraerd A.H., Nicolaï B.M.,
563 Ferguson I. (2011) Where systems biology meets postharvest. *Postharvest Biology and*
564 *Technology*, **62**, 223–237.

565 Ishikawa T., Takahara K., Hirabayashi T., Matsumura H., Fujisawa S., Terauchi R., Uchimiya
566 H., Kawai-Yamada M. (2010) Metabolome analysis of response to oxidative stress in rice
567 suspension cells overexpressing cell death suppressor Bax inhibitor-1. *Plant and Cell*
568 *Physiology*, **51**, 9–20.

569 Kader A.A., Ben-Yehoshua S. (2000) Effects of superatmospheric oxygen levels on
570 postharvest physiology and quality of fresh fruits and vegetables. *Postharvest Biology and*
571 *Technology*, **20**, 1–13.

572 Kader A.A. (2002) Modified atmospheres during transport and storage, in *Postharvest*
573 *Technology of Horticultural Crops*, Ed by Kader AA, University of California, Division of
574 Agriculture and Natural Resources, Davis, CA, USA, pp 135-144.

575 Klok EJ, Wilson IW, Wilson D, Chapman SC, Ewing RM, Somerville SC, Peacock WJ,
576 Dolferus R, Dennis ES (2002) Expression profile analysis of the low-oxygen response in
577 *Arabidopsis* root cultures. *Plant Cell* **14**: 2481–2494

578 Lammertyn J., Franck C., Verlinden B.E., Nicolaï B.M. (2001) Comparative study of the O_2,
579 CO_2 and temperature effect on respiration between 'Conference' pear cell protoplasts in
580 suspension and intact pears. *Journal of Experimental Botany*, **52**, 1769–1777.

581 Lara M.V., Budde C.O., Porrini L., Borsani J., Murray R., Andreo C.S., Drincovich M.F.
582 (2011) Peach (*Prunus Persica*) fruit response to anoxia: reversible ripening delay and
583 biochemical changes. *Plant and Cell Physiology*, **52**, 392–403.

584 Lee J., Rudell D.R., Davies P.J., Watkins C.B. (2011) Metabolic changes in 1-
585 methylcyclopropene (1-MCP)-treated "Empire" apple fruit during storage. *Metabolomics*, **8**,
586 742–753.

587 Lehmann M., Schwarzländer M., Obata T., Sirikantaramas S., Burow M., Olsen C.E., Tohge
588 T., Fricker M.D., Møller B.L., Fernie A.R., Sweetlove L.J., Laxa M. (2008) The metabolic
589 response of *Arabidopsis* roots to oxidative stress is distinct from that of heterotrophic cells in
590 culture and highlights a complex relationship between the levels of transcripts, metabolites,
591 and flux. *Molecular Plant*, **2**, 390–406.

592 Li J., Sun J., Yang Y., Guo S., Glick B.R. (2012) Identification of hypoxic-responsive
593 proteins in cucumber roots using a proteomic approach. *Plant Physiology and Biochemistry*,
594 **51**, 74–80.

595 Magneschi L., Perata P. (2009) Rice germination and seedling growth in the absence of
596 oxygen. *Annals of Botany*, **103**, 181–196.

597 Miyashita Y., Good A.G. (2008) Contribution of the GABA shunt to hypoxia-induced alanine
598 accumulation in roots of *Arabidopsis thaliana*. *Plant and Cell Physiology*, **49**, 92–102.

599 Moing A. (2002) Sugar alcohols as carbohydrate reserves in some higher plants., in: Gupta
600 A.K., Kaur N. (Eds.), Carbohydrate Reserves in Plants – Synthesis and Regulation, Elsevier,
601 pp. 337–358.

602 Norden B., Broberg P., Lindberg C., Plymoth A. (2005). Analysis and understanding of high
603 dimensionality data by means of multivariate data analysis. *Chemistry and Biodiversity*, **2**,
604 1487–1494.

605 Obata T., Fernie A.R. (2012). the use of metabolomics to dissect plant response to abiotic
606 stress. *Cellular and Molecular Life Sciences*, **69**, 3225-3243.

607 Oms-Oliu G., Hertog M.L.A.T.M., Van de Poel B., Ampofo-Asiama J., Geeraerd AH, Nicolaï
608 B.M. (2011) Metabolic characterization of tomato fruit during preharvest development,
609 ripening, and postharvest shelf-life. *Postharvest Biology and Technology*, **62**, 7–16.

610 Pedreschi R., Franck C., Lammertyn J., Erban A., Kopka J., Hertog M., Verlinden B., Nicolaï
611 B. (2009) Metabolic profiling of "Conference" pears under low oxygen stress. *Postharvest*
612 *Biology and Technology*, **51**, 123-130.

613 Pedreschi R., Vanstreels E., Carpentier S., Hertog M., Lammertyn J., Robben J., Noben J.,
614 Swennen R., Vanderleyden J. (2007) Proteomic analysis of core breakdown disorder in
615 Conference pears (*Pyrus communis* L .). *Proteomics*, **7**, 2083–2099.

616 Perez A.G., Sanz C., (2008) Formation of fruit flavour., in: Brückner, B., Wyllie, S.G. (Eds.),
617 Fruit and vegetable flavour: Recent advances and future prospects. Woodhead Publishing
618 Limited, Cambridge, pp. 41-70.

619 Puschmann R., Romani, R. (1983) Ethylene production by auxin-deprived, suspension-
620 cultured pear fruit cells in response to auxins, stress, or precursor. *Plant Physiology*, **73**, 1013-
621 1019.

622 Reggiani R., Aurisano N., Mattana M., Bertani A. (1993) Influence of K^+ ions on polyamine
623 level in wheat seedlings. *Journal of Plant Physiology*, **141**, 136–140.

624 Rios-Estepa R., Lange B.M. (2007) Experimental and mathematical approaches to modeling
625 plant metabolic networks. *Phytochemistry*, **68**, 2351–2374.

626 Roessner U., Wagner C., Kopka J., Trethewey R.N., Willmitzer L. (2000) Simultaneous
627 analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. *Plant*
628 *Journal*, **23**, 131–142.

629 Sachs M.M., Subbaiah C.C., Saab I.N. (1996) Anaerobic gene expression and flooding
630 tolerance in maize. *Journal of Experimental Botany*, **47**, 1–15.

631 Schneegurt M.A., McDaniel C.N. (1986) Amino acid transport in suspension-cultured plant
632 cells. *Plant Physiology*, **81**, 36–40.

633 Schwab W., Davidovich-Rikanati R., Lewinsohn, E. (2008) Biosynthesis of plant-derived
634 flavor compounds. *Plant Journal*, **54**, 712-732.

635 St. Cyr J.A., Bianco R.W., Schneider J.R., Mahoney J.R.Jr., Tveter K., Einzig S., Foker J.E.
636 (1989). Enhanced high energy phosphate recovery with ribose infusion after global
637 myocardial ischemia in a canine model. *Journal of Surgical Research*, **46**,157–162.

638 Shulaev V., Cortes D., Miller G., Mittler R. (2008) Metabolomics for plant stress response.
639 *Physiologia Plantarum*, **132**, 199–208.

640 Sweetlove L.J., Last R.L., Fernie A.R. (2003) Predictive metabolic engineering: a goal for
641 systems biology. *Plant Physiology*, **132**, 420–425.

642 Thomson C.J., Greenway H. (1991) Metabolic evidence for stelar anoxia in maize roots
643 exposed to low O_2 concentrations. *Plant Physiology*, **96**, 1294–1301.

644 Van de Poel B., Bulens I., Oppermann Y., Hertog M.L.A.T.M., Nicolai B.M., Sauter M.,
645 Geeraerd A.H. (2012) S-adenosyl-L-methionine (SAM) usage during climacteric ripening of
646 tomato in relation to ethylene and polyamine biosynthesis and transmethylation capacity.
647 *Physiologia Plantarum*, **148**, 176-188.

648 Wahl S.A., Dauner M., Wiechert W. (2004) New tools for mass isotopomer data evaluation in
649 13C flux analysis: Mass isotope correction, data consistency checking, and precursor
650 relationships. *Biotechnology and Bioengineering*, **85**, 259–268.

651 Williams T.C., Miguet L., Masakapalli S.K., Kruger N.J., Sweetlove L.J., Ratcliffe R.G.
652 (2008) Metabolic network fluxes in heterotrophic *Arabidopsis* cells: stability of the flux
653 distribution under different oxygenationconditions. *Plant Physiology*, **148**, 704–18.

654 Ye B., Muller H.H., Zhang J., Gressel J. (1997) Constitutively elevated levels of putrescine
655 and putrescine-generating enzymes correlated with oxidant stress resistance in *Conyza*
656 *bonariensis* and Wheat. *Plant Physiology*, **115**, 1443–1451.

657

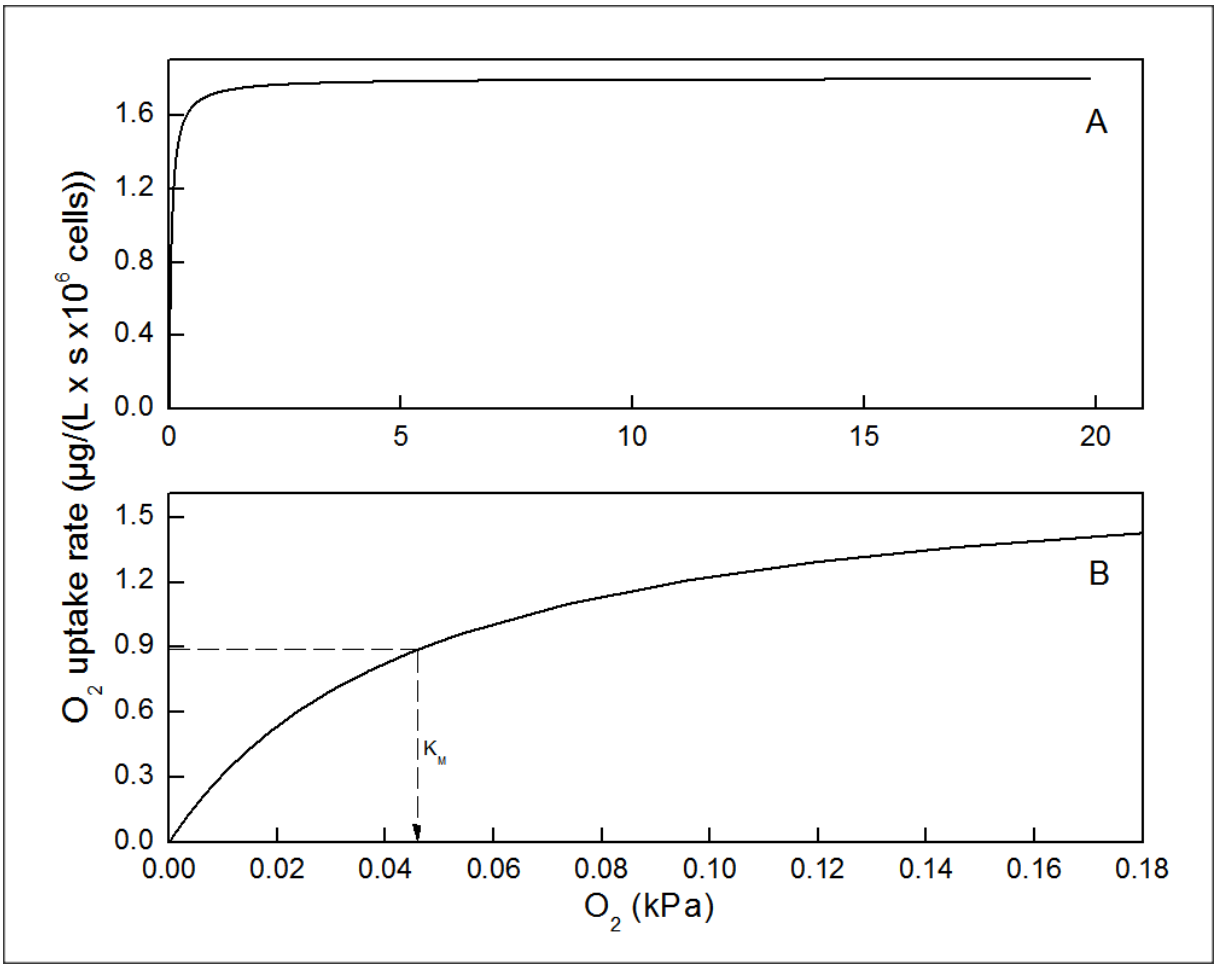
Tables

Table 1. Variable Important Plot (VIP) scores of the top 10 metabolites under the different O_2 conditions and incubation time. VIP's were calculated from the correlation loadings after PLS-DA analysis as explained in materials and method

0 kPa		1 kPa		21 kPa		Time	
Metabolite	VIP	Metabolite	VIP	Metabolite	VIP	Metabolite	VIP
Citramalate	6.916	Citramalate	10.111	Pyroglutamate	1.555	Gluconate	9.519
Valine	2.686	Valine	3.488	Mannose	1.549	Benzoate	5.731
Sorbitol	2.634	Leucine	3.371	Ribose	1.477	GABA	5.636
Leucine	2.583	Sorbitol	3.330	Stearate	1.466	Aspartate	5.619
Pyroglutamate	2.163	Pyroglutamate	2.588	Lactate	1.440	Methionine	5.043
Fructose	1.491	Fructose	1.716	Laurate	1.423	Erythritol	3.312
Tyrosine	1.472	Tyrosine	1.661	Sorbitol	1.419	Glutamate	2.487
Gluconate	1.463	Homoserine	1.489	Maltose	1.399	Lyxose	2.227
Benzoate	1.427	Norleucine	1.473	Shikimate	1.394	β-alanine	2.118
Glycerol-3-P*	1.343	Glycerol-3-P	1.465	Malate	1.389	3-PG#	1.994
Homoserine	1.326	Maltitol	1.424	Glycerate	1.361	Alanine	1.796

*Glycerol-3-phosphate, #3-phosphoglycerate

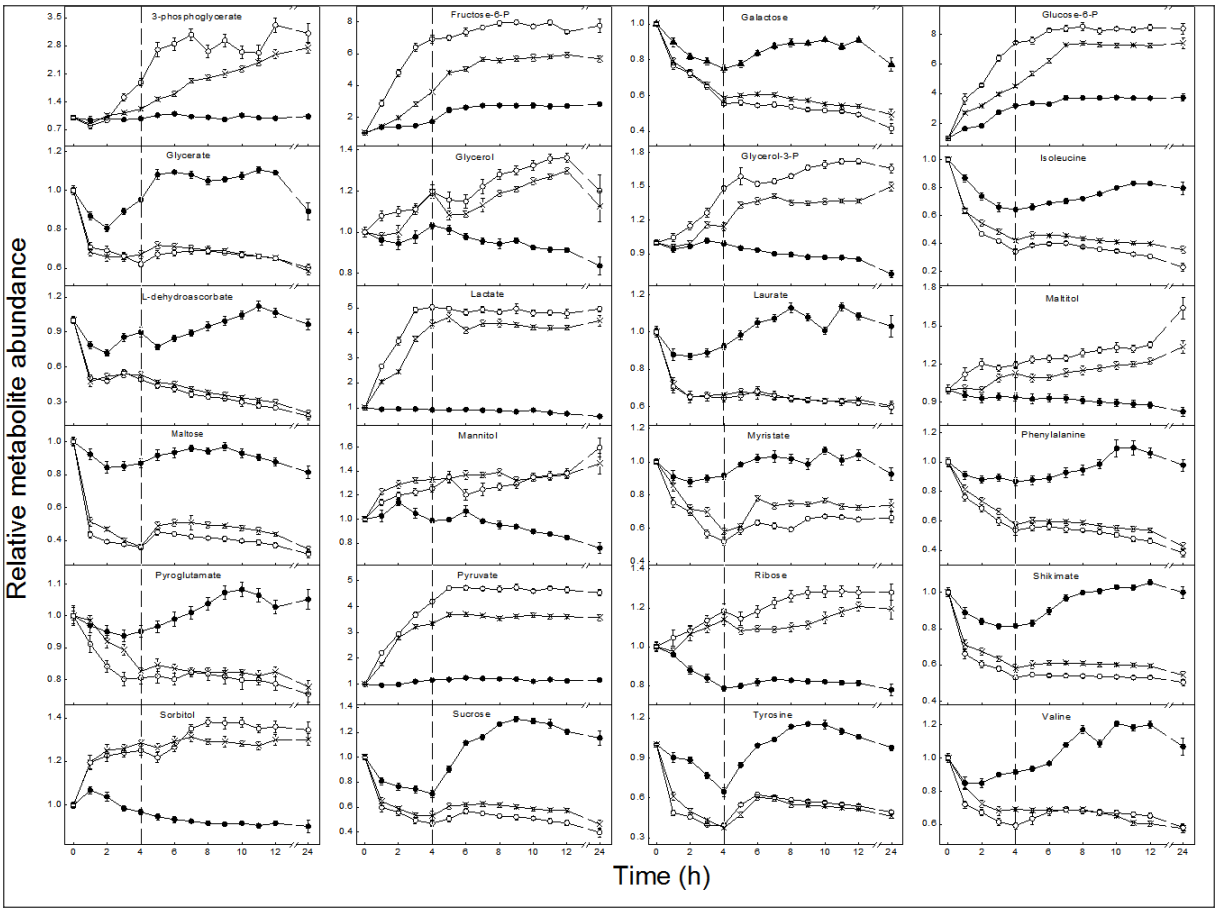
666 **Figure Legends**



667

668 **Fig. 1.** Estimation of the respiration kinetics of cultured cell suspension of *Lycopersicum*
669 *esculentum* (A) Uptake rate of O_2 by tomato cells as a function of O_2 partial pressure (B) A
670 magnified portion of the uptake rate to show the K_M of respiration.

671



672

673 **Fig. 2.** A plot of the 24 most altered metabolites out of the total of 63 reported in Appendix S1
674 following the incubation of tomato cell suspension cultures under different O_2 levels [21 kPa
675 (●), 1 kPa (x) and 0 kPa (○)]. Metabolites are ordered alphabetically (DHAA, L-
676 dehydroascorbate; Fructose-6-P, fructose-6-phosphate; Glucose-6-P, Glucose-6-phosphate).
677 All values are means of four independent replicates expressed relative to starting value. Error
678 bars indicate the standard error of the mean. The dashed lines indicate the time point where
679 ^{13}C labeled glucose was added.

680

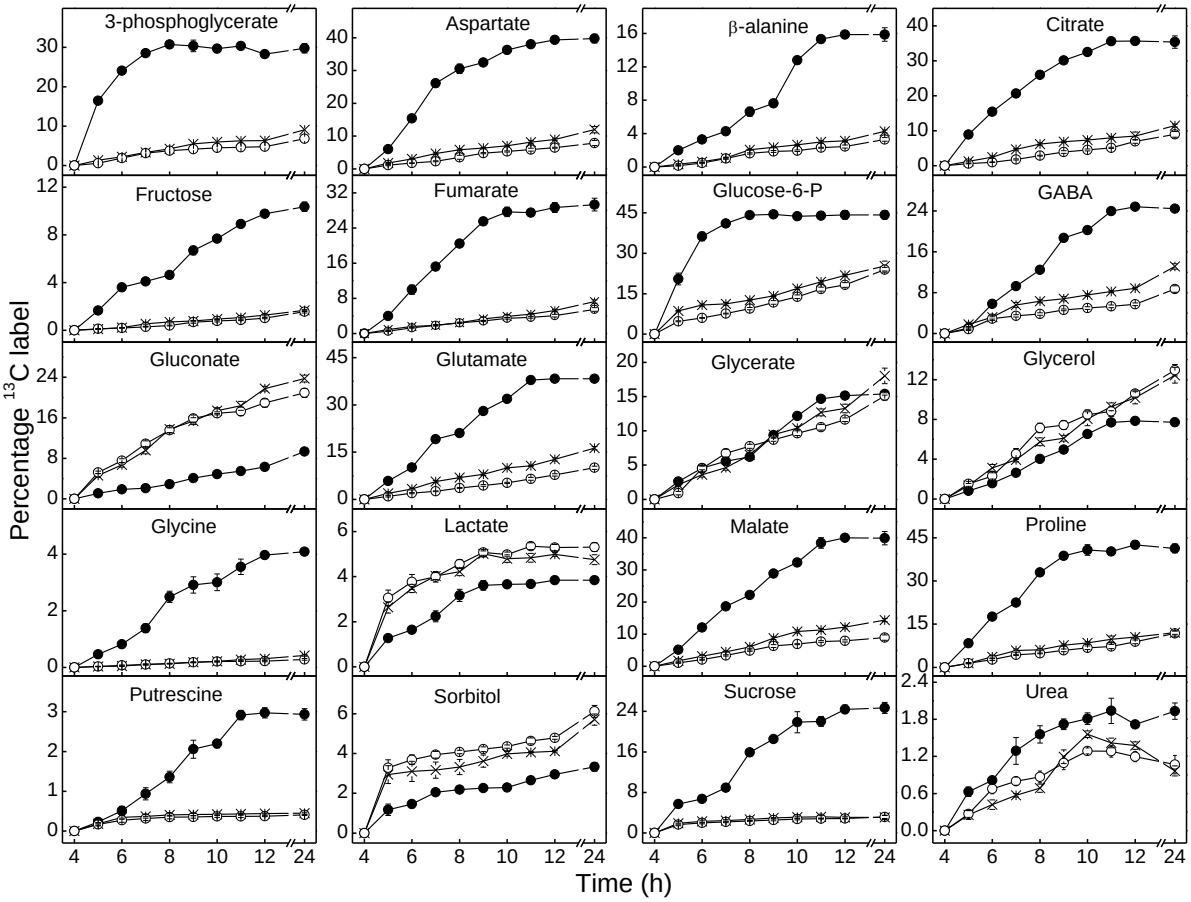
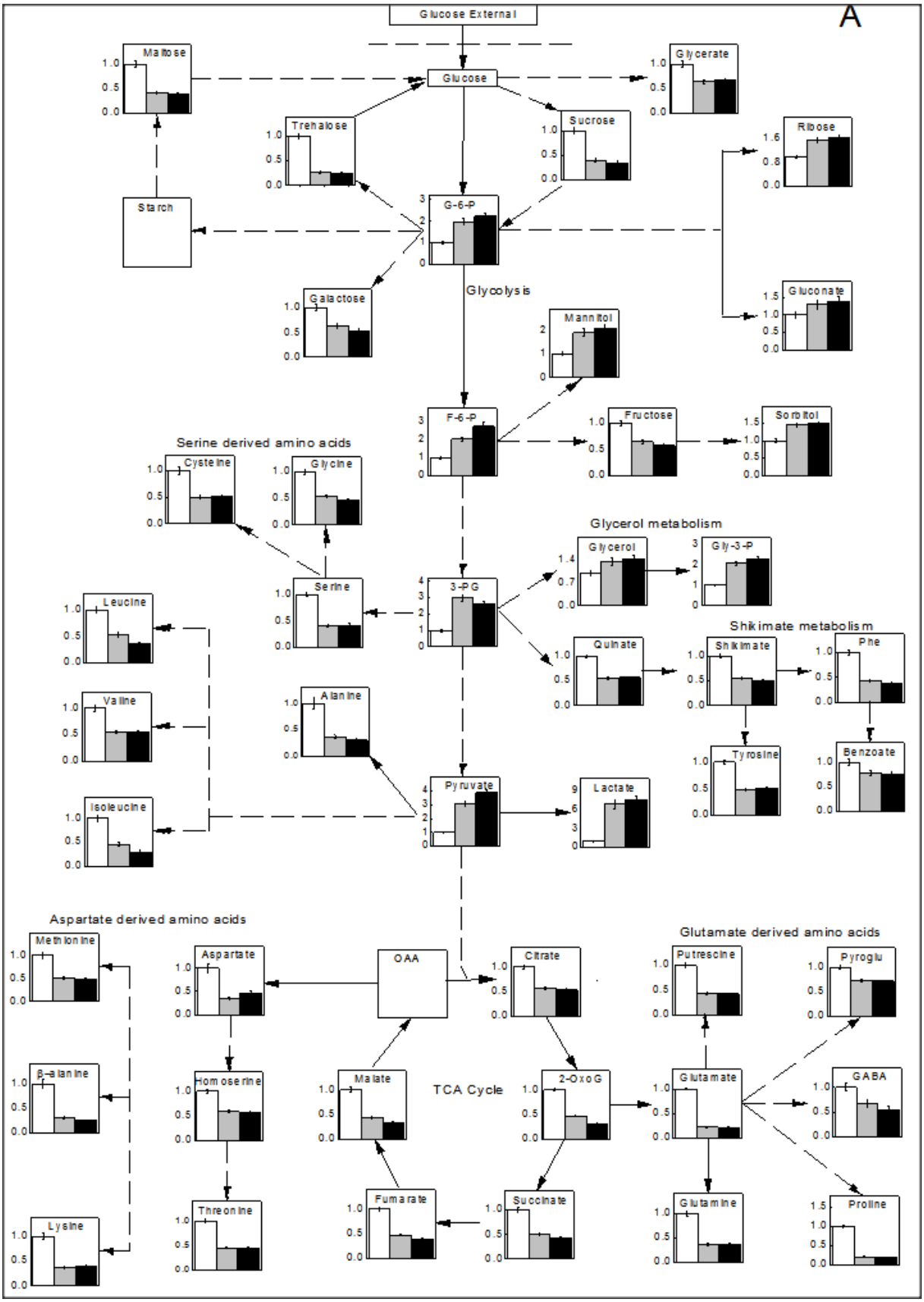


Fig. 3. Accumulation of ^{13}C label by the metabolites shown in Fig. 3 with the exception of DHAA, laurate, maltitol, myristate and pyruvate whose ^{13}C label data could not be obtained. All values are means of four independent replicates. Error bars indicate the standard error of the mean. Labels are 21 kPa (●), 1 kPa (x) and 0 kPa (○). The time axes starts from4 hours, the moment ^{13}C labeled glucose was introduced.


A
Glucose External
Maltose
Glucose
Glycerate
Trehalose
Sucrose
Ribose
Starch
G-6-P
Glycolysis
Galactose
Mannitol
Gluconate
F-6-P
Fructose
Sorbitol
Serine derived amino acids
Cysteine
Glycine
Glycerol metabolism
Glycerol
Gly-3-P
Serine
3-PG
Leucine
Quinate
Shikimate metabolism
Shikimate
Phe
Valine
Alanine
Tyrosine
Benzoate
Pyruvate
Lactate
Isoleucine
Aspartate derived amino acids
Glutamate derived amino acids
Methionine
Aspartate
OAA
Citrate
Putrescine
Pyroglu
β-alanine
Homoserine
Malate
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2-OxoG
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GABA
Fumarate
Succinate
Glutamine
Proline
Threonine
Lysine

688

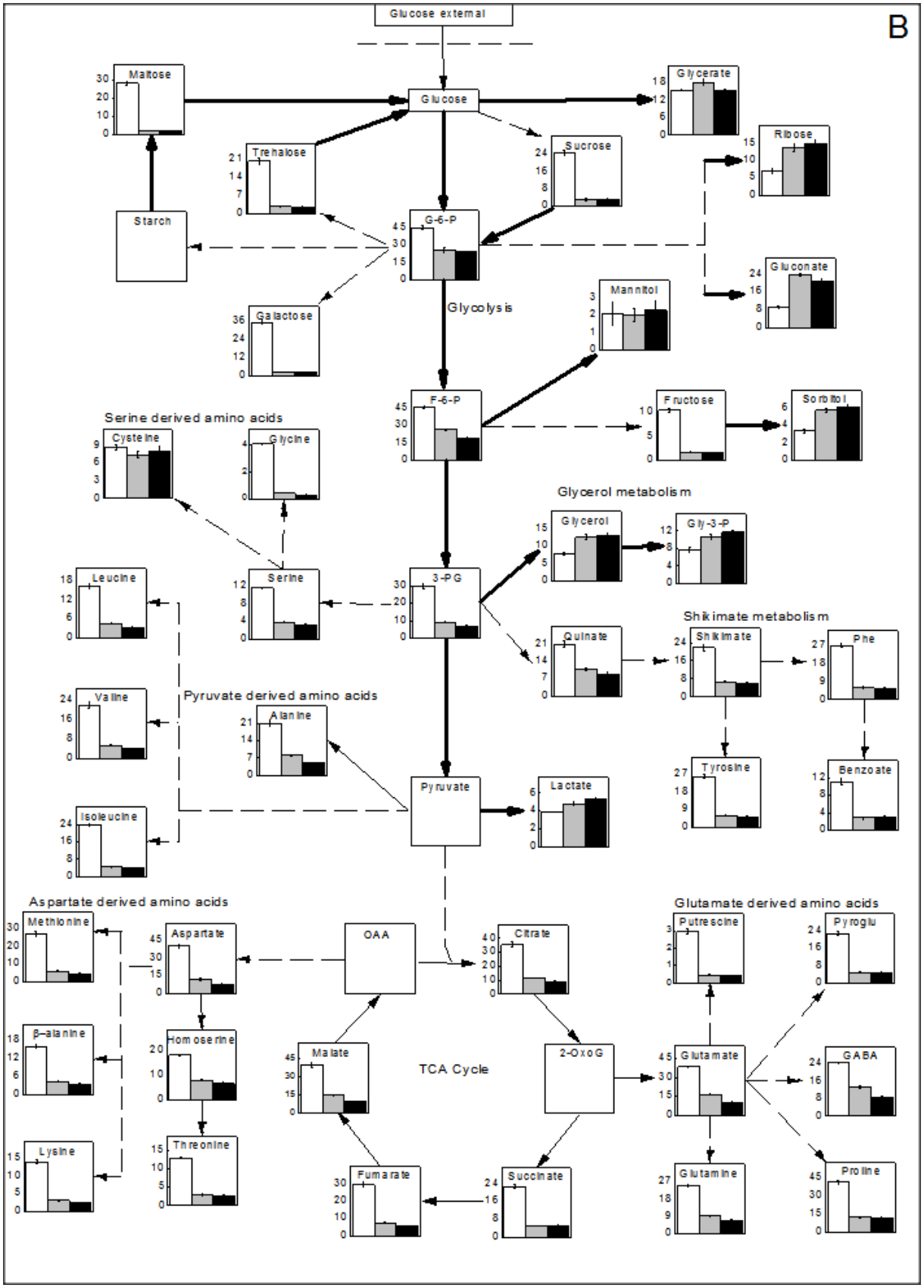
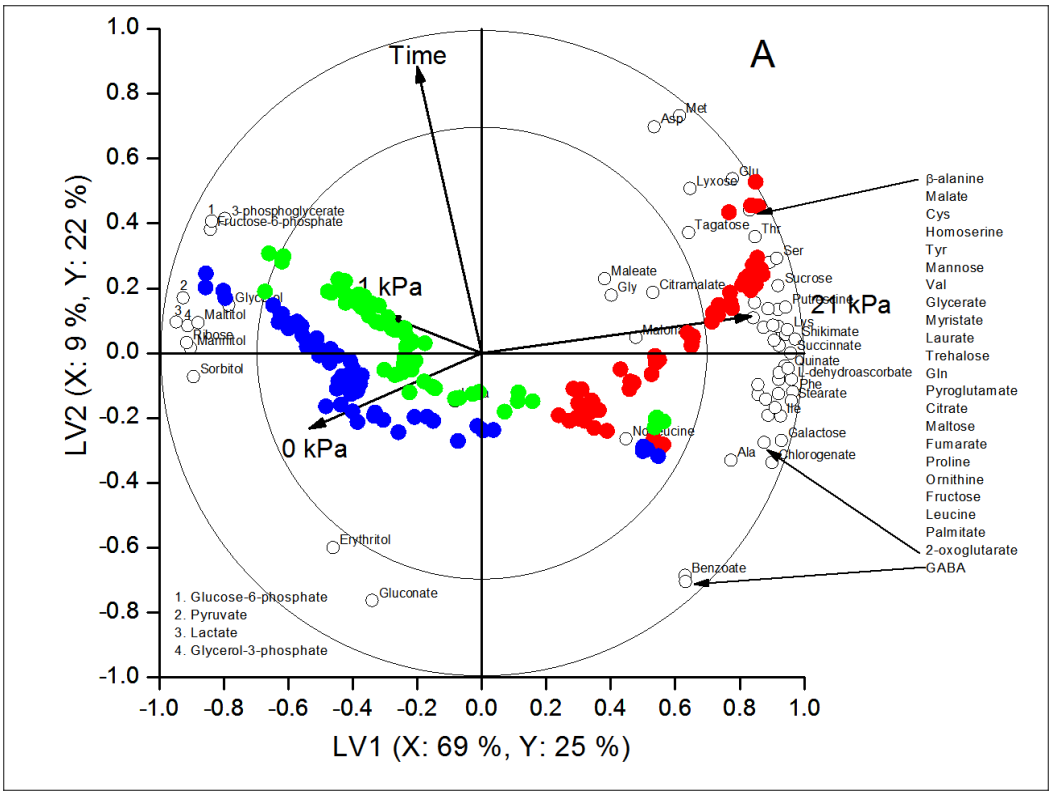


Fig. 4. (A) Metabolic network map showing the changes in relative abundance after 24 h of incubating tomato cell culture under different O_2 levels. (B) Metabolic network map showing

692 the accumulation of ^{13}C label by the different metabolites with the thickness of the arrows
693 increased for pathways which are up-regulated under low O_2 stress. The white, gray and dark
694 bars represent 21 kPA, 1 kPA and 0 kPa O_2 levels, respectively. All values are means of four
695 independent replicates expressed relative to starting value. Error bars indicate the standard
696 error of the mean (2-OxoG, 2-oxoglutarate; 3-PG, 3-phosphoglycerate; OAA, oxaloacetate;
697 Phe, phenylalanine; Pyroglu, pyroglutamate).
698



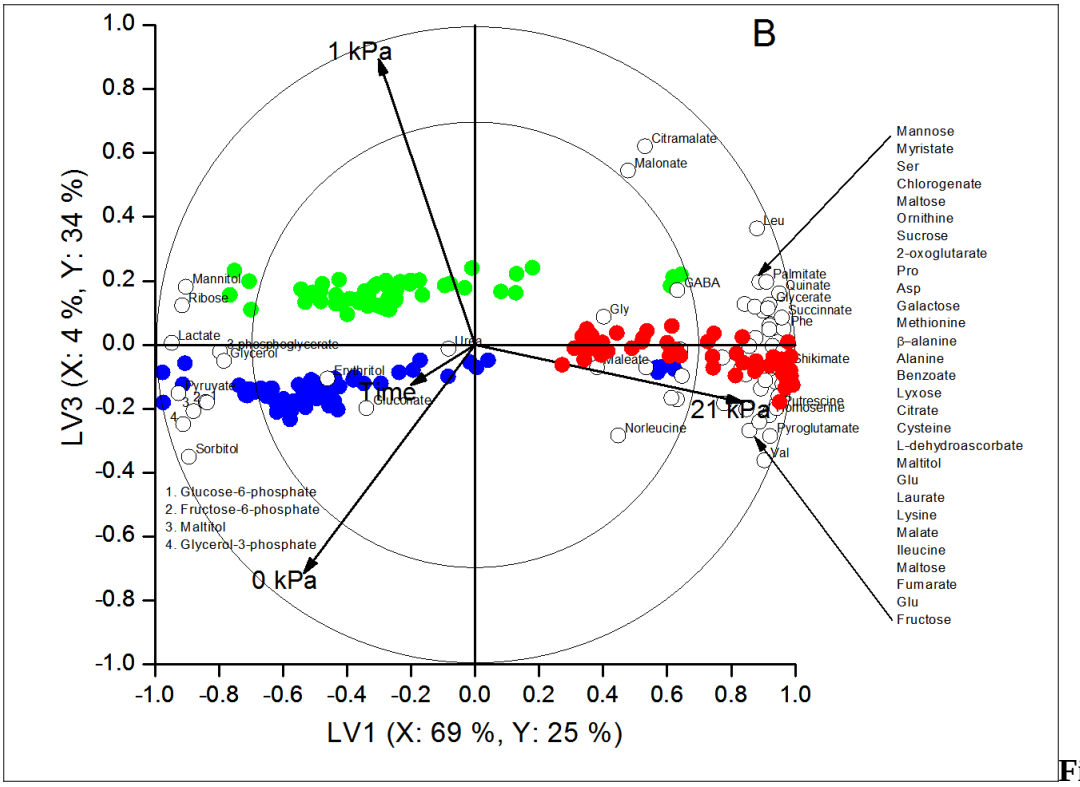


Fig. 5. PLS-DA bi-plot showing tomato cells samples taken from the O_2 conditions at different time points. Figures (A) and (B) show a plot of latent variables (LV) 1 vs. LV2 and LV1 vs. LV3 respectively. The percentage explained variance (X and Y representing the O_2 level and

metabolite abundance respectively) are inserted next to the LV. The samples scores (closed circles: red, 21 kPa; green, 1 kPa; blue 0 kPa) are superimposed on the metabolite loadings (open circles). The line arrows indicate the position of the treatments (O_2 levels and incubation time) in the new parameter space. Amino acids are represented by their three letter symbols.